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Laboratory diet influences cold tolerance in a genotype-dependent manner in *Drosophila melanogaster*

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ABSTRACT

Cold stress can reduce insect fitness and is an important determinant of species distributions and responses to climate change. Cold tolerance is influenced by genotype and environmental conditions, with factors such as day length and temperature having a particularly strong influence. Recent studies also indicate that diet impacts cold tolerance, but it is unclear whether diet-mediated shifts in cold tolerance are consistent across distinct genotypes. The goal of this study was to determine the extent to which commonly used artificial diets influence cold tolerance in *Drosophila melanogaster,* and whether these effects are consistent across genetically distinct lines. Specifically, we tested the impact of different fly diets on 1) ability to survive cold stress, 2) critical thermal minimum (CT_{min}), and 3) the ability to maintain reproduction after cold stress. Experiments were conducted across six isogenic lines from the *Drosophila* Genetic Reference Panel, and these lines were reared on different fly diets. Cold shock survival, CT_{min}, and reproductive output pre- and post-cold exposure varied considerably across diet and genotype combinations, suggesting strong genotype by environment interactions shape nutritionally mediated changes in cold tolerance. For example, in some lines cold shock survival remained consistently high or low across diets, while in others cold shock survival ranged from 5% to 75% depending on diet. Ultimately, these results add to a growing literature that cold tolerance is shaped by complex interactions between genotype and environment and inform practical considerations when selecting a laboratory diet for thermal tolerance experiments in *Drosophila.*

1. Introduction

Meeting nutritional demands in the face of fluctuating food availability poses a significant environmental challenge, necessitating changes in behavior, physiology, and/or life-history strategies to cope (reviewed in McCue, 2010). In insects, diet composition influences fitness-related traits such as growth, immune function, stress resistance, fecundity, and longevity (Burger et al., 2007; Colinet et al., 2013; Frost et al., 2010; Kim et al., 2020; Littlefair et al., 2017; Littlefair and Knell, 2016; Mbande et al., 2020; Valtonen et al., 2012; Vijendravarma et al., 2010; Xia and de Belle, 2016). While many studies have addressed the effects of varying caloric content on life history traits (e.g., Burger et al., 2007; Edgar, 2006; Koyama et al., 2013), dietary composition, independent of caloric content, can also have important influences on life history and physiology (Andersen et al., 2010; Clark et al., 2015; Henry et al., 2020; Kim et al., 2020; Montoro et al., 2020). Current views on nutritional ecology, such as the Geometric Framework of Nutrition, indicate that organisms require precise ratios of nutrients to maximize fitness (Raubenheimer et al., 2009; Raubenheimer and Simpson, 2018). Supporting this framework, several studies have shown diets lacking key nutrients or containing unbalanced nutrient content can cause significant reduction in growth, reproduction, immunity, and survival (Cotter et al., 2019; Henry et al., 2020; Kim et al., 2020; Montoro et al., 2020; Ng et al., 2019).

In insects, dietary composition can have strong effects on cold tolerance, an important ecological trait that is tightly linked to species distribution (Andersen et al., 2010; Burger et al., 2007; Colinet et al., 2013; Colinet and Renault, 2014; Henry et al., 2020; Jiménez-Padilla et al., 2020; Kim et al., 2020; Shreve et al., 2007). In nature, cold stress varies in its duration and severity, and different types of cold stress can have distinct effects on physiology and fitness (e.g., locomotor defects, decreased fecundity, mortality; Colinet et al., 2015; Garcia and Teets,

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2019; Marshall and Sinclair, 2010). The link between temperature and fitness in insects mediates species distribution and population structure, the ability to invade novel habitats, and responsiveness to global climate change (Addo-Bediako et al., 2000; Bellard et al., 2013; Kellermann et al., 2012a, 2012b; Rosenberger et al., 2017; Youngsteadt et al., 2017; Williams et al., 2015). Further, several recent studies in *D. melanogaster* have shown distinct cold tolerance traits are independent of one another and vary across distinct genotypes (Garcia et al., 2020; Gerken et al., 2016; Gerken et al., 2015; Teets and Hahn, 2018). Thus, precise effects of diet on cold tolerance may depend on the particular trait being measured.

Previous research indicates that carbohydrate-rich diets tend to improve cold tolerance relative to protein-rich diets (Andersen et al., 2010; Burger et al., 2007; Colinet et al., 2013; Henry et al., 2020), possibly a result of providing substrates for the accumulation of cryoprotectants such as polyols, sugars, and free amino acids (Colinet et al., 2013; Koštál et al., 2011; Olsson et al., 2016; Overgaard et al., 2007; Overgaard and Macmillan, 2017; Storey and Storey, 2012; Vesala et al., 2012). Consumption of a carbohydrate-rich diet triggers considerable changes in metabolic and lipidomic profiles – e.g., increasing body sugar levels – which could explain the apparent relationship between carbohydrate-rich diets and the ability to survive in the cold (Colinet et al., 2013; Enriquez and Colinet, 2019). However, a causal link between diet-induced osmolyte accumulation and insect cold tolerance has not been established (reviewed in Overgaard and Macmillan, 2017). Conversely, protein-rich diets typically improve reproductive output relative to carbohydrate-rich diets, likely by increasing protein pools for egg production and vitellogenesis (Burger et al., 2007; Goane et al., 2019; Jensen et al., 2015; Kim et al., 2020; Lee, 2015; Littlefair et al., 2017; Trajković et al., 2017). The hormone signals needed for oogenesis (e.g., ecdysone and juvenile hormone), as well as downstream processes that occur during oogenesis, are also highly dependent upon dietary proteins (reviewed in Mirth et al., 2019; Wheeler, 1996). Insufficient dietary protein impedes egg chamber development (Drummond-Barbosa and Spradling, 2001) and reduces production of egg-yolk proteins by the fat body and follicle cells (Bownes and Reid, 1990). Potential nutrient and energetic tradeoffs between reproduction and stress tolerance suggest that protein-rich diets may reduce cold tolerance (Chen et al., 2017; Marshall and Sinclair, 2010; Sinclair, 2015; Stazione et al., 2020), although this prediction has not been empirically demonstrated. Further, unlike carbohydrates and proteins, dietary lipids tend to benefit both cold tolerance and reproductive output, providing energetic resources and specific lipid components that support cell structure and function (Arrese and Soulages, 2010; Cambron et al., 2019; Potts et al., 2020; Shreve et al., 2007; Ujvari et al., 2009).

The studies described above demonstrate that dietary composition can significantly affect insect stress physiology and fecundity. However, the majority of these studies use genetically admixed populations (e.g., Andersen et al., 2010) or a single genetic background (e.g., Burger et al., 2007). Thus, it is unclear whether dietary effects on life history traits are consistent across genotypes. The few studies that have considered intraspecific variation indicate there are genotype by environment interactions in nutritionally mediated traits. Kim et al. (2020) found that dietary composition differentially impacts longevity, development, and fecundity across six genetically distinct strains of *Drosophila melanogaster,* while Thompson (2019) found that diet-induced plasticity in allometric scaling between body parts of a grasshopper (*Melanoplus sanguinipes*) is influenced by heritable genetic variation. However, whether dietary mediated changes in cold tolerance depend on genetic background has not been addressed.

D. melanogaster has been a workhorse for cold tolerance research (e.g., Colinet et al., 2015; Garcia et al., 2020; Garcia and Teets, 2019; Marshall and Sinclair, 2010; Overgaard and Macmillan, 2017; Sinclair et al., 2015; Teets and Hahn, 2018), and a practical consideration for fly research is the choice of a laboratory diet, with most labs selecting a diet based on a combination of convenience, lab tradition, and the species/lines being propagated. These diets can differ substantially in nutrient content, particularly protein to carbohydrate ratios, suggesting that the choice of a laboratory diet could influence the results of thermal tolerance experiments. In addition to direct effects on trait values, genotype by environment interactions for diet-mediated phenotypes could also influence the rank-order of traits across genotypes. The results of genetic association studies and other mechanistic studies often rely on rank-order for analysis (e.g., Teets and Hahn, 2018; Lecheta et al., 2020), so if rank order across lines is sensitive to diet, then results may not be comparable across studies or they may lack field relevance. For example, two of our previous studies (Teets and Hahn, 2018; Garcia et al., 2020) assessed cold shock survival across several common lines from the *Drosophila* Genetic Reference Panel (DGRP; Mackay et al., 2012), and we observed that survival differed between these studies, although rank order among lines was similar. While most rearing conditions (e.g., temperature, humidity, light cycle) were kept constant between the two studies, different artificial diets were used. While this comparison suggests reasonable consistency in the rank-order of cold tolerance across two laboratory diets, it is uncertain whether this pattern would hold true across a wider range of diets and cold tolerance metrics. Thus, a deeper investigation of the influence of laboratory diet on stress-related traits is needed to inform discussions on best practices for thermal tolerance studies in *Drosophila*.

In this study we addressed two specific objectives: 1) determine the extent to which laboratory diet affects cold tolerance and reproduction across genetically distinct strains of *D. melanogaster* and 2) determine whether the rank-order of trait values across lines depends on diet. We reared six isogenic lines on four commonly used artificial diets that vary in their nutritional content (Table 1). Across all line/diet combinations, we measured cold shock survival, critical thermal minimum (CT_{min}), and fecundity before and after sublethal cold exposure. These traits are well defined and validated metrics of insect cold tolerance (Sinclair et al., 2015) and have been shown to be genetically independent of one another (Garcia et al., 2020). Previous work on cold acclimation indicates that the capacity for cold tolerance plasticity shows considerable genetic variation (e.g., Gerken et al., 2015). Considering this, we hypothesize that the degree of diet-mediated plasticity in cold tolerance and reproduction will depend on genetic background. Accordingly, if diet-mediated plasticity varies across genotypes, we also predict that the rank order of cold tolerance will depend on diet. Further, while not a specific goal of the study, by selecting diets with widely varying nutrient content, we indirectly tested the hypothesis that specific dietary components support certain traits while hindering others.

2. Methods

2.1. Laboratory diets and nutritional content estimation

We selected four diets commonly found throughout the fly literature. For simplicity, we identify these diets as Soy-flour (SF), Banana media (BM), a standard Cornmeal-Molasses diet (CM1), and a nutrient-rich Cornmeal-Molasses variant (CM2) (Table 1; See Suppl. Table 1 for recipes). While CM1 and CM2 share similar ingredients, the quantity of those ingredients greatly differs. We estimated nutritional content (caloric and macronutrient content) of each diet by summing the nutritional content of each individual ingredient according to information available through the United States Department of Agriculture's FoodData Central database (https://fdc.nal.usda.gov/index.html).

2.2. Fly strains and rearing

For these experiments, we used six lines from the Drosophila Genetic Reference Panel (DGRP), whose cold tolerance was determined in a prior study (Garcia et al., 2020). The chosen lines have either high, average, or low cold tolerance based on the ability to survive a cold shock at −2 °C for 1 h, with two lines per category (Suppl. Table 2). Flies

Table 1
Estimated Dietary Caloric and Macronutrient Content. Provided are the estimated caloric content, macronutrient content, and ratio between macronutrients for each diet used. All dietary information was acquired from the USDA FoodData Central database (https://fdc.nal.usda.gov/index.html) and given as amount per 1 L of water. We provided sources for each recipe when available, otherwise we provided a citation to works which have previously used the recipe.

Diet	Calories (kcal/L)	Protein (g/L)	Carbohydrate (g/L)	Lipid (g/L)	P:C:L Ratio	Source
Banana Media (BM)	495.3	15.6	113.3	2.8	8:42:1	Brazner and Etges, 1993
Cornmeal-Molasses (CM1)	487.8	13.1	101.3	5.4	3:21:1	Drosophila Species Stock Center
Cornmeal-Molasses Variant (CM2)	818.7	44.1	149.5	12.3	4:12:1	Lockwood et al., 2018
Soy-Flour (SF)	598.3	18.5	122.9	7.4	3:18:1	Bloomington Drosophila Stock Center

Table 2
Results from statistical analyses testing the effects of diet, line, and sex on cold tolerance. Table indicates the effects of each independent variable and their interactions on cold-shock survival, CT_{min}, and cold-induced changes in fecundity. For cold-induced changes in fecundity, exposure (pre vs. post) has been abbreviated to "Exp." * denotes significance ($p < 0.05$). For survival we used a nominal logistic regression, for CT_{min} we used a Cox hazard analysis, and for cold-induced changes in fecundity we used a general linear regression.

Survival	DF	L-R χ^2	*p*-Value
Diet	3	97.40	<0.0001*
Line	5	1095.65	<0.0001*
Sex	1	40.60	<0.0001*
Diet × Line	15	226.81	<0.0001*
Diet × Sex	3	68.31	<0.001
Line × Sex	5	19.83	0.0001*
Diet × Line × Sex	15	54.85	<0.0001*

CTmin	DF	L-R χ^2	p-Value
Diet	3	1.70	0.64
Line	5	102.31	<0.0001*
Sex	1	11.48	0.0007*
Diet × Line	15	62.10	<0.0001*
Diet × Sex	3	1.28	0.73
Line × Sex	5	22.49	0.0004*
Diet × Line × Sex	15	69.73	<0.0001*

Fecundity	DF	Sum. Sq.	F-Value	p-Value
Diet	3	20.47	6.82	<0.0001*
Line	5	27.30	5.46	<0.0001*
Exp. (pre v. post)	1	0.007	0.007	0.935
Diet × Line	15	38.78	2.59	<0.0001*
Diet × Exp.	3	140.20	49.73	<0.0001*
Line × Exp.	5	73.92	14.78	<0.0001*
Diet × Line × Exp.	15	82.81	5.52	<0.0001*

used for experiments were reared on the indicated diets for two generations to eliminate influence from prior rearing conditions and maternal effects. We supplemented all diets with live baker's yeast to promote egg laying and reduce the proliferation of wild yeast, which can influence insect cold tolerance (Colinet and Renault, 2014). Second generation adult flies (5–8 days after eclosion) maintained at 25 °C, 65–75% relative humidity, and 12:12 L:D were used for all experiments. To generate flies for experiments, we placed 3–4 males and 6–8 females from each strain into vials filled with ~5 ml of diet and allowed them to lay eggs for 3 d. The resulting first-generation progeny (3–4 males and 6–8 females) were placed on fresh diet and allowed to lay for 3 d. The offspring from these flies, which were the second generation of flies reared on the variable diets, were transferred from their initial rearing vials into fresh vials 1–3 d after emergence and allowed to mate for 24 h. After 24 h, we lightly anesthetized flies with CO_2 (between 5 and 10 min under anesthesia), sorted by sex, and transferred them to their experimental vials. Flies were allowed to recover from anesthesia for at least 48 h before being used in experiments. The number of flies used varied between experiments and are detailed below.

2.3. Acute cold shock

We transferred groups of flies into 50 ml falcon tubes, used cotton to restrain them to the lower third of the tube, and submerged tubes in an A24B programmable refrigerated bath (Thermo Scientific, Waltham, MA, USA). We exposed flies to −2 °C for 1 h before transferring to new food vials to recover for 24 h. After the 24 h recovery, we counted the number of live individuals. Flies that could right themselves spontaneously or in response to gentle prodding were considered alive. We separately measured males and females in groups of 20. In total, we measured 8 groups (4 male and 4 female) per diet/strain combination spread evenly across two cohorts.

2.4. CT_{min}

We placed flies into a temperature controlled, jacketed column (300 mm long × 50 mm diameter) at 25 °C, as described in Awde et al. (2020). We pumped a chilled water/propylene glycol mixture from a programmable bath into the jacketed column, cooling the column at a rate of 0.25 °C/min. Temperature within the column was continuously recorded using a TC-08 Omega Thermocouple module (Omega, Norwalk, CT, USA). CT_{min} was recorded as the temperature at which flies lost neuromuscular function and fell into a collection vial below. As flies fell, they passed through a custom-built infrared counter (Trikenetics, Whatman, MA. USA), which logged the time the fly fell. We cross-referenced the time a fly fell against the temperature of the column at that time to obtain the temperature at which a fly fell (CT_{min}). We separately assayed males and females in groups of 20. In total, we measured 40 individuals (20 males and 20 females) per diet/strain combination.

2.5. Fecundity pre- and post-cold exposure

We isolated mated females onto their respective diets and allowed them to lay eggs for 24 h. Afterwards, we transferred the female to a fresh vial and placed them into an incubator set at 4 °C for 8 h before allowing them to recover from additional 24 h. From a pilot study we found this exposure had low mortality rates while also eliciting significant changes in reproductive output. After recovery, we transferred the exposed female to another fresh vial of their respective diet and allowed them to lay for a final 24 h. We monitored and recorded emergence of adult progeny from the pre- and post-exposure vials daily. We measured 18 females per diet/strain combination spread evenly across five cohorts.

2.6. Statistical analysis

We performed all statistical analysis in R (v. 3.6.3) implemented in R Studio (v. 1.1.463.; R Studio Team 2015). We performed a logistic regression using the *"lme4"* package to analyze cold shock survival and a Cox Hazard analysis using the *"survival"* package to analyze CT_{min}. For both analyses, strain, diet, sex, and their interactions were used as fixed effects. For the logistic regression, we used replicate nested within treatment group as a random effect to avoid psuedoreplication due to non-independence among individuals within each vial. To analyze

changes in reproduction before and after a sub-lethal cold exposure, we performed a general linear mixed model with a log-link function using the *"lme4"* package. We included strain, diet, time (pre and post), and their interactions as fixed effects and individual as a random effect.

3. Results

For cold shock survival, we found that line, diet, sex, and their interactions all had significant effects on survival (Table 2; Fig. 1a–b). Because of the challenges in interpreting the three-way interactions, we also ran the analyses separately for each sex (*Suppl. Table 3*), and the line*diet interaction was significant for both sexes, indicating that the effect of diet on cold shock survival depends on genotype. This line*diet interaction can be highlighted by a few specific results. First, for example, some lines had significant variation in survivorship across diets (e.g., DGRP-313, which varied from 3 ± 2% to 75 ± 7% survival across the four diets in both sexes), while others had almost no change in survivorship across diets (e.g., DGRP-208 in both sexes). Second, no single diet consistently resulted in higher or lower cold shock survival relative to other diets in both males and females (Fig. 1a–b).

For CT_{min}, line, sex, diet*line, line*sex, and diet*line*sex all had significant effects (Table 2; Fig. 2a–b). Once again, we analyzed data separately for each sex, and for both females and males, the line*diet interaction was significant (*Suppl. Table 3*). While the results were largely similar for both sexes, in some cases the CT_{min} of females was more sensitive to a change in diet (Fig. 2a–b). For example, males from DGRP-379 had a consistent CT_{min} across diets (ranging from 5.19 ± 0.33 to 6.00 ± 0.46 °C) while CT_{min} in DGRP-379 females considerably varied across diets (ranging from 3.28 ± 0.30 to 5.78 ± 0.31 °C). As with cold shock tolerance, no single diet consistently raised or lowered CT_{min} relative to the other diets across all line and sex combinations (Fig. 2a–b).

For cold-induced changes in fecundity, we found significant effects of diet, line, diet*line, diet*exposure, and diet*line*exposure (Table 2, Fig. 3a–d). Surprisingly, cold exposure alone had no effect on fecundity. However, several interaction terms containing cold exposure were significant, indicating that cold exposure does affect fecundity in combination with other factors. Each line appears to have variable responses to cold exposure depending on diet. However, we did observe one general trend across lines. Prior to cold exposure, lines reared on the CM2 diet tended to have higher fecundity than when reared on the other diets (Fig. 3c), having nearly double the 24 h fecundity (number of

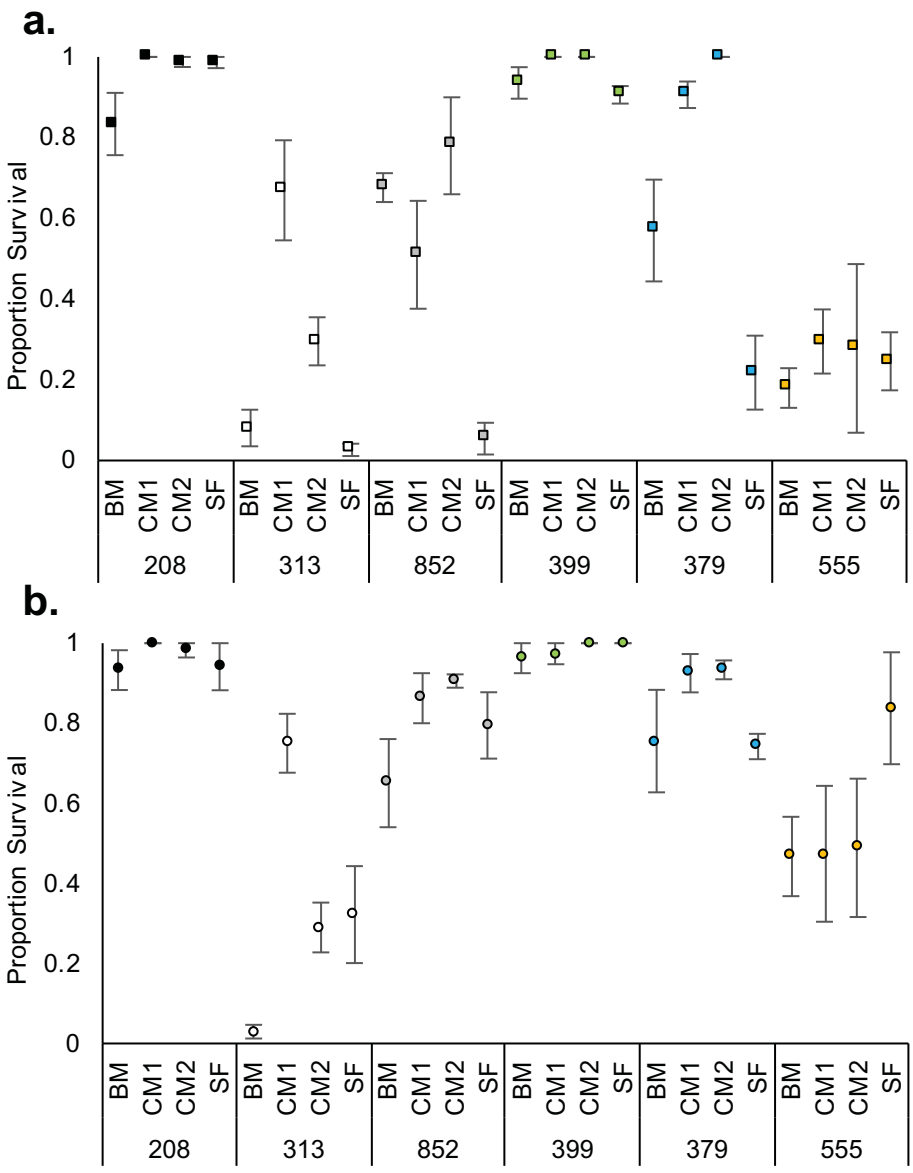

Fig. 1. *Variation in cold-shock survival across line, diet, and sex.* Panels illustrate the interactive effect between diet and line on cold-shock survival in (a) females and (b) males. Symbols indicate fitted means and S.E.M. Each line is color coded, and numbers along the x-axis indicate the DGRP line number. Diet abbreviations are as follows: BM = banana media, CM1 = cornmeal-molasses recipe 1, CM2 = cornmeal-molasses recipe 2, and SF = soy flour recipe. See Methods and Supplementary Material for a description of the diets.

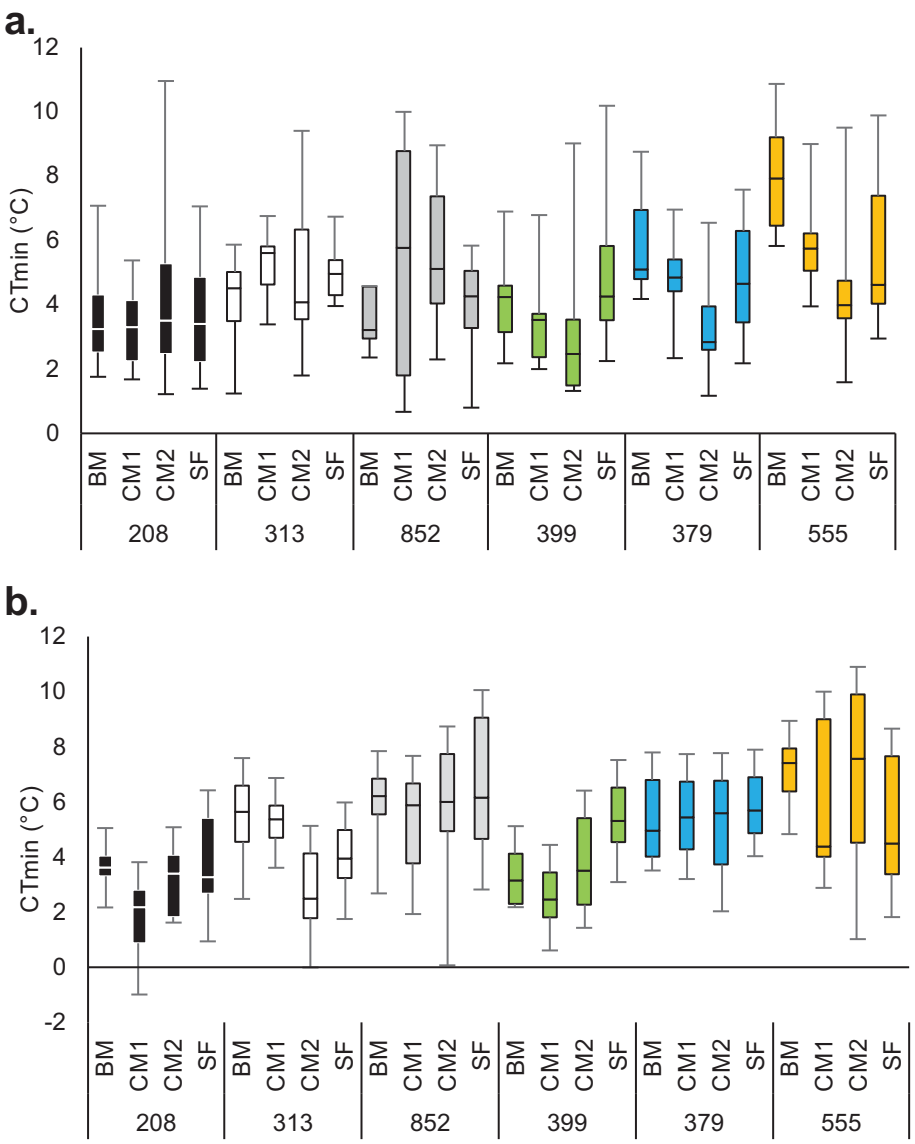

Fig. 2. *Variation in CT_{min} across line, diet, and sex.* Box and whisker plots illustrate the interactive effect between diet and line on cold-shock survival in (a) females and (b) males. Plots were generated from the raw data for each group. Each line is color coded, and numbers along the x-axis indicate the DGRP line number. Diet abbreviations are as follows: BM = banana media, CM1 = cornmeal-molasses recipe 1, CM2 = cornmeal-molasses recipe 2, and SF = soy flour recipe. See Methods and Supplementary Material for a description of the diets.

offspring = 10 ± 1 per female) relative to the remaining diets (range: 4.8 ± 0.5 and 6.5 ± 0.6). However, most lines reared on the CM2 diet exhibited a significant drop in fecundity following cold exposure (Δ = −5.3), while lines reared on the other diets exhibit either had no change (SF: Δ = 0.75), a slight decrease (BM: Δ = −1), or a slight improvement in fecundity following cold exposure (CM1: Δ = 2.6).

To determine the extent to which diet influences the rank-order of traits across lines, we ranked all lines from 1 (best) – 6 (worst) for each trait across all diets, using the fitted means derived from our statistical models. We ranked males and females separately for cold-shock survival and CT_{min}. We assessed the stability of each trait as follows: 1) We first calculated the average rank for each line across the four diets. 2) We then calculated how much each line deviated from its average rank for each diet. 3) Finally, we took the average deviance across all 24 line*diet combinations. A low average deviance would indicate a trait with a stable rank order, while a high average deviance would indicate a trait with an unstable rank order. We found trait rank order was labile across diets, but the exact lability depended on trait and sex (Fig. 4a-f). Rank order for cold-shock survival and CT_{min} was more consistent in males (average deviance = 0.46 & 0.75, respectively; Fig. 4 a,c) relative to females (average deviance = 0.77 & 1.08, respectively; Fig. 4b, d). In females, baseline fecundity was the least consistent trait (average deviance = 1.16; Fig. 4e) relative to all other traits measured while cold-induced changes in fecundity was the most consistent trait (average deviance = 0.71; Fig. 4f).

4. Discussion

Here, we quantified the effects of laboratory diet on cold tolerance and fecundity across six genetically distinct lines of *D. melanogaster*. First, we tested the hypothesis that variation in nutrient content would influence cold tolerance and fecundity, and that this diet-induced plasticity would vary across genetic backgrounds. Our results support this hypothesis, with cold shock survival, CT_{min}, baseline fecundity, and cold-induced changes in fecundity varying as a function of diet in a genotype-dependent manner. Responses to diet varied across lines, with some lines showing no variation in cold shock survival and CT_{min} across diets, while other lines showed significant variance in cold shock survival and CT_{min} across diets. Second, we assessed the extent to which the rank order of cold tolerance varied as a function of diet and trait measured. Across diets, the stability of rank-order depended on sex and trait measured. Trait rank order in males was more consistent relative to females, and for both sexes, the rank order of cold shock survival tended to be the most stable relative to other traits. Lastly, we found no single

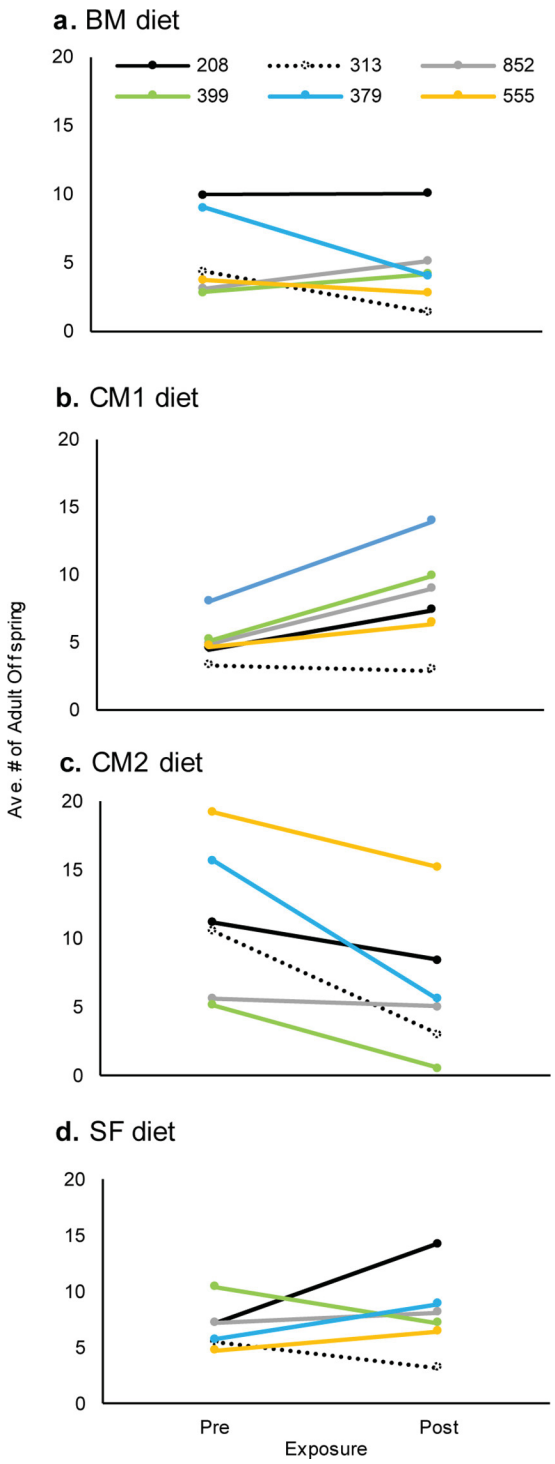

Fig. 3. *Interactive effect of line and diet on fecundity following a sub-lethal cold exposure.* Panels illustrate changes in 24 h fecundity following cold stress for a) BM diet, b) CM1 diet, c) CM2 diet, and d) SF diet. Graphs were generated using fitted means, and for ease of viewing, error bars have been removed. Different colors represent different diet*line combinations. Diet abbreviations are as follows: BM = banana media, CM1 = cornmeal-molasses recipe 1, CM2 = cornmeal-molasses recipe 2, and SF = soy flour recipe. See Methods and Supplementary Material for a description of the diets.

dietary composition improved or reduced cold tolerance across lines in either sex. Fecundity was highest for most lines when reared on a nutrient-rich diet, but flies on this diet also had the greatest declines in fecundity following exposure to a sub-lethal cold stress. Overall, our results demonstrate that dietary variation can lead to significant changes in fitness-related traits, but that these changes are influenced by genetic background.

Dietary composition can influence fitness-related traits in insects including growth, development, fecundity, immunity, and stress physiology (Andersen et al., 2010; Burger et al., 2007; Colinet et al., 2013; Colinet and Renault, 2014; Cotter et al., 2019; Henry et al., 2020; Jiménez-Padilla et al., 2020; Kim et al., 2020; Shreve et al., 2007; Thompson, 2019). Diet-induced plasticity in life-history also varies across genetic backgrounds, indicating nutritionally mediated phenotypes follow a classic G × E interaction (Kim et al., 2020; Thompson, 2019). Our findings are consistent with this, showing that fecundity significantly differed as a function of both diet and genetic background, and their interaction. While previous work has shown that stress tolerance traits show plastic responses to diet, it was unclear whether there is a genetic component to this diet-induced plasticity. Here we measured two well established cold tolerance traits, cold shock survival and CT_{min} (Sinclair et al., 2015), and found both varied as a function of diet and genetic background. We found cold shock survival was highly sensitive to diet in some lines, while in other lines survival was consistent across diets and sexes. For some of the consistent lines, survival was nearly 100% across all diets, which leaves the possibility that diet-induced variation in cold shock survival may become apparent at lower temperatures. Within lines that had plastic changes in cold tolerance across diets, no single diet consistently improved or reduced cold shock survival in either sex. Likewise, CT_{min} varied across diet and line combinations, but again we found no single diet that consistently changed CT_{min} in either direction for either sex. Our findings contribute to a growing literature that diet-induced plasticity in fitness-related traits (e.g., growth, development, reproduction, cold tolerance) is influenced by underlying genetic variation (Kim et al., 2020; Thompson, 2019).

The DGRP used in this study is primarily used as a tool for Genome Wide Association Studies (GWAS). Even if trait values change across conditions, reliable genetic associations can be obtained if there is fidelity in the rank-orders. Thus, we evaluated the extent to which the rank order of our traits changed across diets. The rank-order of all traits was influenced by diet, although the relative lability of these rank orders varied across traits and sexes. The rank order of cold-shock survival was more consistent than that of CT_{min}. When factoring in sex, trait rank order across diet was more consistent in males relative to females for both cold-shock survival and CT_{min}, likely due to sex-specific differences in investment trade-offs between cold tolerance and fecundity (Chen et al., 2017; Marshall and Sinclair, 2010; Sinclair, 2015; Stazione et al., 2020). In females, rank order for baseline fecundity was the most labile trait, while cold-induced changes in fecundity was the least labile. Considering these findings, we recommend using cold shock survival to rank cold tolerance for genetic associations, especially if the stability of rankings across studies is a concern (e.g., Teets and Hahn, 2018). That said, our previous work indicated that distinct cold tolerance traits lack genetic correlation (Garcia et al., 2020), and that there is no "true" measure of cold tolerance when comparing across isogenic lines. Thus, genetic associations for other metrics like CT_{min} still have value, provided investigators recognize these associations may be context and/or sex dependent (e.g. Lecheta et al., 2020). We also note that our sample size of lines is small ($N = 6$), so additional experiments are needed to fully determine the extent to which diet influences the rank order of traits across lines.

While we did not systematically vary nutrient composition across diets, we observed some interesting trends that contribute to discussions on how genetic background can influence diet-mediated plasticity in fitness-related traits. Carbohydrate-rich diets tend to improve insect cold tolerance relative to protein-rich diets (Andersen et al., 2010; Burger et al., 2007; Colinet et al., 2013), a trend proposed to be mediated by carbohydrate-induced changes in metabolomic and lipidomic profiles (Colinet et al., 2013; Enriquez and Colinet, 2019). Our findings are partly consistent with these works. Flies reared on the carbohydrate-

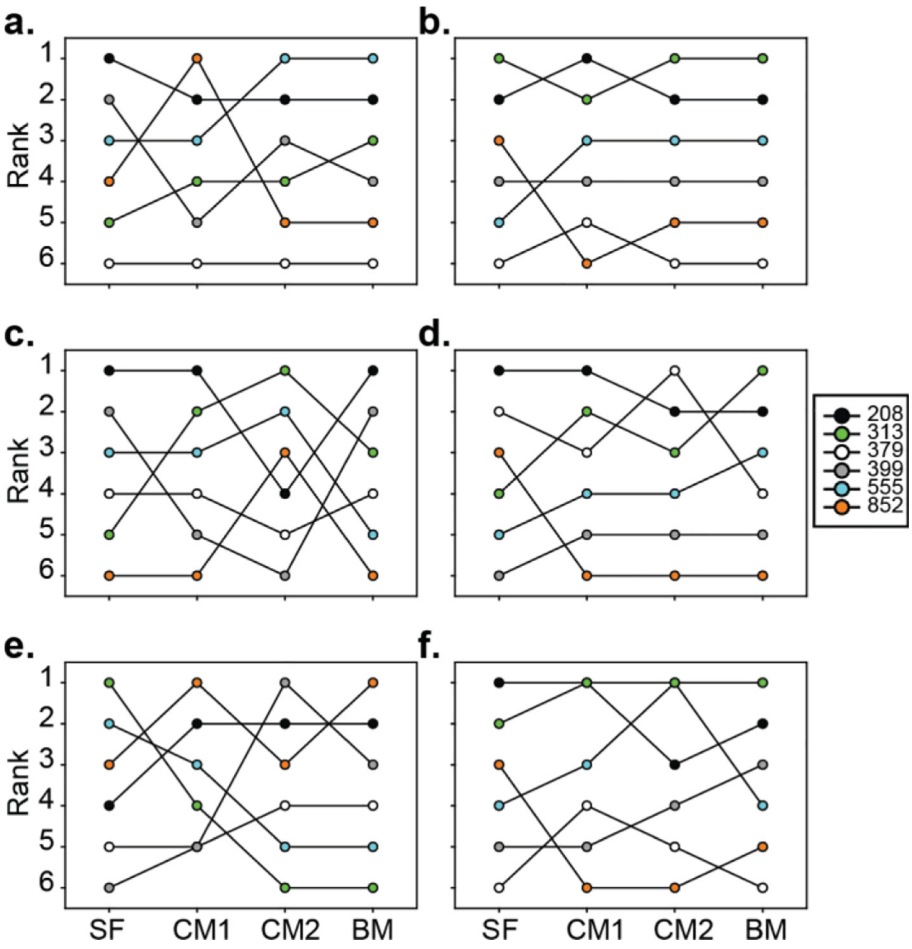

Fig. 4. *Dietary influence on trait rank order across lines.* Lines were ranked best (1) to worst (6) for cold-shock survival (a-b), CT_{min} (c-d), baseline fecundity (e), and cold-induced changes in fecundity (f) using fitted averages from our statistical models. Cold-shock survival and CT_{min} were ranked separately for females (a,c) and males (b,d). Line numbers indicate DGRP line numbers, and diet abbreviations are as follows: BM = banana media, CM1 = cornmeal-molasses recipe 1, CM2 = cornmeal-molasses recipe 2, and SF = soy flour recipe. See Methods and Supplementary Material for a description of the diets.

rich CM2 diet had improved cold shock survival relative to flies reared on the carbohydrate-poor BM and CM1 diets. While the carbohydrate-poor SF diet also elicited improved cold shock survival, the CM2 and SF diets both have higher lipid concentrations, and previous work has shown lipids are an important energy source during stress and that specific classes of lipids preserve membrane function in the cold (Potts et al., 2020; Shreve et al., 2007; Zhu et al., 2017). In addition to macronutrient content, caloric content can also influence cold tolerance (Burger et al., 2007; Henry et al., 2020). Henry et al. (2020) found that flies reared on low calorie diets have higher cold shock survival, but we observed the opposite, with flies reared on the high-calorie CM2 and SF diets tending to have higher cold shock survival. Finally, our diets likely vary in certain micronutrients, and previous work has demonstrated that cold tolerance may benefit from certain micronutrients that could assist in maintaining ion homeostasis during or after cold stress. In both *D. melanogaster* and the field cricket (*Gryllus pennsylvanicus*), increased dietary sodium consumption improves some measures of cold tolerance (i.e., CCR), but not others (i.e., CTmin; Lebenzon et al., 2020; Yerushalmi et al., 2016).

Our work also highlights an important role for dietary protein in regulating fecundity. Dietary proteins are important for several aspects of oogenesis, from signals mediating egg production to development of egg casings and yolk (reviewed in Mirth et al., 2019; Wheeler, 1996), and as a result protein rich-diets tend to support increased fecundity (Burger et al., 2007; Goane et al., 2019; Jensen et al., 2015; Kim et al., 2020; Lee, 2015; Littlefair et al., 2017; Trajković et al., 2017). In our experiments, flies reared on the protein rich CM2 diet had the highest reproductive output relative to the remaining three diets, further demonstrating the importance of dietary proteins for reproduction. The CM2 diet has the most balanced C:P:L ratio relative to the others, which also supports higher reproductive output (Henry et al., 2020; Kim et al., 2020; Lee, 2015). However, we must note that the CM2 diet has the highest concentrations of all three macronutrients relative to the others (which is mostly due to substantially higher inactive yeast content than the other diets) and thus, we cannot unequivocally contribute the observed improvements in fecundity solely to dietary proteins. Flies reared on the CM2 diet also had the greatest decline in reproductive output following exposure to sub-lethal cold stress, while flies on other diets had little to no changes in fecundity following stress. One limitation of the fecundity experiments is that fecundity was scored as the number of adult offspring produced by each female, but we did not assess egg-to-adult viability on each of the diets. Thus, differences in egg-to-adult viability across diets could influence our measurements of absolute fecundity, although this limitation would not confound our ability to assess relative changes in reproduction following cold stress. Further, the stark differences in fecundity observed across lines and diets are unlikely to be explained by differences in egg-to-adult viability. Taken together, our results suggest that when nutrients are abundant (i.e., in the CM2 diet), most lines allocated resources to reproduction, but this high allocation to reproduction makes flies more susceptible to stress-induced reductions in fecundity. However, when nutrients are more limited, the relationship between fecundity and cold stress across genotypes is less predictable.

5. Conclusions

We demonstrated here that there is a significant intraspecific variation in diet-induced plasticity for both cold tolerance and fecundity.

Each genotype had a distinct response to variation in diet, with some exhibiting significant diet-mediated plasticity in cold tolerance or fecundity, and others not. This result indicates that diet-induced plasticity is likely heritable and thus has evolutionary potential. Our findings also have practical considerations for laboratory studies of stress tolerance in *D. melanogaster*. First, the DGRP lines we used for these experiments have been used in several cold tolerance studies (Garcia et al., 2020; Garcia and Teets, 2019; Gerken et al., 2015; Lecheta et al., 2020; Teets and Hahn, 2018), and one concern with these lines is that inbreeding depression could limit the field relevance of results. However, it does not appear than any lines consistently performed poorly across all diets, which might be expected if inbreeding depression were driving variation across lines. Rather, our rank-order analyses (Fig. 4) indicate that in some cases, trait values are more dependent on diet, which should be considered when comparing different lines in the DGRP. Second, several metrics are used to measure insect cold tolerance (Sinclair et al., 2015), but which metric(s) best approximates an insect's actual cold tolerance is still unclear (see Andersen et al., 2015; Chown et al., 2009). We recently showed that commonly used metrics (e.g., CTmin, Chill Coma Recovery, Cold Shock Survival) are genetically distinct from one another (Garcia et al., 2020), and here we expand on those findings by showing that these traits (both absolute trait values and rank-order) also vary as a function of diet. However, some traits are more stable than others across diet, and based on our results, we recommend that future studies consider cold shock survival if a stable rank-order is essential. As a final practical consideration, we recommend that future studies should provide explicit dietary details. In the cold tolerance literature, there is minimal discussion of dietary conditions, specific diet recipes are seldom provided, and the original source of the diet recipe is often absent. Our results indicate that explicit details on diet are needed to facilitate comparison across studies and to promote future studies interested in how dietary composition influences insect stress physiology.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2021.110948.

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